<table>
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<th>Mean (SD) or n (%)</th>
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<td><strong>FENO</strong></td>
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<tr>
<td>Allergy</td>
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<td>Hay fever</td>
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<td>Grassmix spec. IgE</td>
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</tr>
</tbody>
</table>

1 exposure at follow-up compared to baseline
2 mean (SD) for continuous variables and n (%) for categorical variables
3 compared to less exposure; adjusted for age, gender, smoking and farm childhood
4 categorical variable
5 geometric mean and standard deviation
ABSTRACT

Rationale: Studies in men and mice have shown that farm dust exposure induces high levels of IL-17 in the airways, airway neutrophilia and airway hyperresponsiveness (AHR). In addition, it has been shown that agricultural workers have a high prevalence of respiratory symptoms related to nonallergic asthma. In nonallergic asthma, neutrophilic inflammation in the airways is predominant and this phenotype is associated with poor corticosteroid responsiveness. Since IL-17 is known to induce airway neutrophilia and has been associated with corticosteroid resistant airway inflammation, we hypothesized that farm dust exposure induces airway neutrophilia and AHR that are both resistant to corticosteroid treatment.

Methods: Dust extracts were prepared from dust samples collected at cattle farms. Female Balb/c mice (n=6-8 per group) were exposed to farm dust extract (FDE), house dust mite (HDM, model for allergic airway inflammation) or PBS (negative control), via intranasal instillation 4 times per week during six weeks. During the last three weeks, mice received intranasal instillation of fluticasone propionate (FP) or vehicle two hours before the antigen or PBS exposures. AHR to methacholine was assessed by flexiVent® 24 hours after the last exposure. Inflammatory cell numbers were assessed in bronchoalveolar lavage fluid (BALF) and lung tissue and cytokines in BALF.

Results: FDE-exposure induced AHR, which was accompanied by more neutrophils in BALF and lungs, more macrophages in the lungs and higher levels of CXCL1, IL-1β, IL-6, IL-17 and TNFα in BALF as compared to PBS-exposed mice. Interestingly, FP treatment reduced FDE-induced AHR and levels of CXCL1, IL-1β and IL-17 whereas it did not affect neutrophil counts, IL-6 and TNFα levels. In the allergic airway inflammation model, FP treatment reduced HDM-induced AHR, and IL-4, IL-5 and IL-13 levels in BALF and numbers of eosinophils in BALF and lungs. Parameters that best predicted AHR varied depending on the animal model used: in the FDE model, IL-1β and IL-17 were the best predictors of AHR, while in the HDM model TNFα and IL-6 accounted the most for the variability of AHR.

Conclusions: FDE-exposure induced corticosteroid resistant lung neutrophilia in mice, whereas in contrast to our hypothesis, FDE-induced AHR and IL-17 were prevented by corticosteroid treatment. This suggests that although IL-17 is important in inducing airway neutrophilia, it may not be required for sustained neutrophil infiltration. Moreover, our results show that AHR in mice is multi-factorial and, depending on the disease setting, different (inflammatory) parameters contribute to its presence and severity.
INTRODUCTION
Asthma is a chronic inflammatory disease of the airways characterized by recurrent airflow obstruction and airway hyperresponsiveness. Clinically, asthma is classified into two main phenotypes: allergic and nonallergic asthma. Allergic asthma is typically a T helper 2 type (Th2) driven disease, with increased levels of interleukin (IL)-4, IL-5 and IL-13, and consequent eosinophilia, increased mucus production and sensitization to specific allergens. Nonallergic asthma is usually more severe than the allergic phenotype and is less responsive to corticosteroid treatment (1,2). Albeit that the pathophysiology of nonallergic asthma is not fully understood, several studies have shown involvement of chemokine (C-X-C motif) ligand 1 (CXCL1), IL-17, and neutrophils (3,4,5).

It has been postulated that a major proportion of asthma in the general population is nonallergic (5). In studies investigating work-related (occupational) asthma, this phenomenon has been mostly referred to as the asthma-like syndrome, and has been show to be highly prevalent in farmers. (5,6). Farm exposures have been shown to constitute a risk factor for development of other pulmonary manifestations, including chronic bronchitis, chronic obstructive pulmonary disease (COPD) (7) and nonatopic wheeze (8). We and others have previously shown in mice and men that farm-related exposures are associated with respiratory symptoms (8) and induce nonallergic lung inflammation, mainly composed of neutrophils and macrophages, and high levels of IL-17 (9-12).

Airway hyperresponsiveness (AHR) is a common feature of allergic and nonallergic asthma, although it is not specific to asthma. Although the mechanisms of AHR are still largely unknown, several factors have been shown to contribute to it, including inflammatory cells and mediators, airway smooth muscle thickening and collagen deposition around the airways, as well as neuronal mechanisms controlling airway tone (13).

Since IL-17 has been shown to be involved in corticosteroid resistance (14,15) and AHR (14), we hypothesized that IL-17 contributes significantly to corticosteroid resistant lung neutrophilia and AHR following exposure to farm dust extract (FDE) in mice. To tackle this hypothesis, FDE-exposed mice were treated with fluticasone propionate and effects on AHR, lung inflammation and IL-17 levels were assessed. The effects of corticosteroid treatment on FDE-induced nonallergic inflammation were compared to the effects on allergic airway inflammation using our well-established house dust mite mouse model (9,16). In addition, we investigated the main drivers of AHR in our nonallergic and allergic asthma mouse models.

MATERIAL AND METHODS
Farm dust extract (FDE)
Settled dust samples were collected from 30 cattle farms. Dust was pooled and FDE was prepared as described previously (9). Briefly, aliquots of ~2.5 gram pooled dust were mixed for 5 min with 12.5 gram glass beads and sterile water to complete a volume of 50 ml in a Greiner tube, on an end-over-end roller at room temperature. Samples were sonicated for 5 min in an ultrasonic bath with crushed ice and aliquots were pooled in a glass Erlenmeyer, with sterile water added to a final concentration of 10 mg dust per ml,
and sodium chloride solution till a final concentration of 0.9%. Erlenmeyers were shaken for 6 hours at room temperature. Then samples were centrifuged at 20,000g/4°C/45 min and subsequently the dust extract was dialyzed (MWCO 3500) against DI-distilled water, sterilized by filtration through a 0.22 um filter and lyophilized.

**Animals**

Female specific pathogen free Balb/c mice aged 6-8 weeks (Harlan, Horst, The Netherlands) were housed in groups of 6-8 mice each and had access to standard food and water *ad libitum*. Mice were exposed to farm dust extract (FDE), house dust mite (HDM, whole bodies from *Dermatophagoides pteronyssinus*, Greer Laboratories, Lenoir, USA, positive control) or PBS (negative control) via nasal instillation 4 times per week during six weeks, following established protocols (9). Briefly, mice were anesthetized with isoflurane and intranasally exposed to FDE (50 µg/day in 50 µl PBS), HDM (40 µg/day in 50 µl PBS) or sterile PBS (50 µl/day). During the last three weeks of exposure, mice were anesthetized with isoflurane and received intranasal instillation of Fluticasone Propionate (FP, Sigma Aldrich, Zwijndrecht, The Netherlands, 10µg/day in 50 µl PBS) or vehicle (50 µl of 2% DMSO solution) two hours before the antigen or PBS exposure (figure 1). Table 1 shows the number of animals per experimental group. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen (Permit Number: 6615A) and procedures were performed under strict governmental and international guidelines.

**Flexivent**

After 6 weeks of exposure, airway hyperresponsiveness was assessed in 6 mice per group by measuring airway resistance (R in cmH2O.s/mL) in response to intravenous (i.v.) administration of increasing doses of methacholine (acetyl-b-methylcholine chloride, Sigma-Aldrich), as previously described (17). Briefly, mice were anesthetized by intraperitoneal injection of a mixture of 100 mg/kg ketamine (Pfizer, New York, USA) and 1mg/kg domitor (Pfizer). After tracheotomization using a 20-gauge intravenous cannula (BD biosciences, Breda, the Netherlands) and cannulization of the jugular vein, mice were attached to a small-animal ventilator; the FlexiVent (SCIREQ, Montreal, Canada), and then paralyzed (i.v. injection of 4 µg rocuronium bromide, Fresenius Kabi, Zeist, The Netherlands, twice during the measurement). Ventilation was adjusted at a breathing frequency of 280 breaths/minute with a tidal volume of 10mL/kg, with pressure limited at 300mmH2O. Anesthesia was maintained by means of supplemental administration of 25% isoflurane and intravenously infusion of a pseudorandom pressure wave. When analyzing the peak resistance and peak compliance, dosages of methacholine was calculated from the pressure response to a 2-second administration of increasing doses of methacholine (acetyl-b-methylcholine chloride, Sigma-Aldrich), as previously described (17).
frequency of 280 breaths/minute with a tidal volume of 10mL/kg, with pressure limited at 300mmH₂O. Anesthesia was maintained by means of supplemental administration of 25% of the initial dose at 20-minute intervals. Airway resistance in response to increasing dosages of methacholine was calculated from the pressure response to a 2-second pseudorandom pressure wave. When analyzing the peak resistance and peak compliance, all values with a coefficient of determination (COD)-value below 0.75 were excluded.

Bronchoalveolar lavage fluid (BALF)

When airway hyperresponsiveness measurements were finished, lungs were lavaged three times with cold sterile PBS and BALF was collected. Total BALF cell numbers was determined using a pocH-100i hematology analyzer (Sysmex, Mundelein, USA), and after centrifugation at 300 xg for 10 minutes, supernatants were collected and stored at -80°C for cytokine analysis. BALF cells were resuspended in RPMI (BioWhittaker Europe, Verviers, Belgium) for preparation of cytopsins. Around 50,000 cells were centrifuged onto glass slides using a cytopsin 4 (Thermo Scientific, Waltham, USA), and a Giemsa staining (Sigma Aldrich) was performed to count differential BALF cells. Percentages of eosinophils, neutrophils, lymphocytes and macrophages were calculated in a total of 300 cells counted.

Concentrations of interferon (IFN)γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, Tumor necrosis factor (TNF)α and CXCL1 were measured in BALF supernatant with a ProcartaPlex™ multiplex ELISA system (eBioscience, Vienna, Austria) on a Luminex 100 system using Starstation software (Applied Cytometry Systems, Sheffield UK).

Table 1. Number of mice per experimental group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Flexivent/BALF/FACS (N)</th>
<th>Histology (N)</th>
</tr>
</thead>
<tbody>
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<td>6</td>
<td>8</td>
</tr>
<tr>
<td>HDM</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>FDE</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>HDM+FP</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>FDE+FP</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Tissue collection

8 mice per group were anaesthetized and sacrificed by cardiac exsanguination. Right lung lobes were carefully inflated with 0.9 ml 50% Tissue Tek, O.C.T. (SaKura, Alphen aan den Rijn, The Netherlands) in PBS, and three of the four lobes were snap frozen and stored at -80°C until use, while the smallest right lung lobe was formalin-fixed and embedded in paraffin. From 6 mice per group, lungs were removed and kept in cold PBS for lung cell isolation.
Lung cell isolation

Lungs were minced and incubated for 45 min at 37°C in RPMI medium supplemented with 10% fetal calf serum (both Lonza, Verviers, Belgium), 10 μg/ml DNase I (grade II from bovine pancreas, Roche Applied Science, Almere, Netherlands), and 0.7 mg/ml collagenase A (Sigma-Aldrich) in a shaking water bath. After that, the digested lung tissue was passed through a 70 μm nylon strainer (BD Biosciences) to obtain single cell suspensions. To lyse contaminating erythrocytes, cell suspensions were incubated with 10 times diluted Pharmlyse (BD Biosciences). Cells were centrifuged through 70 μm strainer caps and counted using a poch-100i hematology analyzer (Sysmex), before they were used for flow cytometry.

Flow cytometric analysis

Single lung cell suspensions were stained for T-cell subsets using the following directly labeled antibodies: APC/Cy7-conjugated anti-CD3 (Biolegend, Fell, Germany), PE/Cy7-conjugated anti-CD4 (Biolegend), PE-conjugated anti-CD25 (Biolegend), FITC-conjugated anti-Foxp3 (eBioscience). Isotype control was used for Foxp3 staining (FITC-conjugated rat IgG2a, eBioscience). Pacific blue-conjugated anti-F 4/80 (Biolegend) and FITC-conjugated anti-CD4 (Biolegend), PE-conjugated anti -CD25 (Biolegend), FITC-conjugated labeled antibodies: APC/Cy7-conjugated anti-CD3 (Biolegend, Fell, Germany), PE/Cy7-

Histology

Sections (3 μm) of formalin-fixed and paraffin-embedded lung tissue were stained for goblet cells, collagen III and α-smooth muscle actin (α-SMA). Goblet cells were stained with Periodic Acid Schiff's (PAS) and all PAS-positive cells in the section were counted manually. The length of all airways was measured at the basal end of the airway epithelium using Aperio ImageScope viewing software 11.2.0.780 (Aperio, Vista, USA), and the total number of PAS-positive cells was expressed per mm airway.

Collagen III was stained using a goat anti-collagen III antibody (Southern Biotech, Alabama, USA), and α-SMA was stained using a mouse anti-α-SMA antibody (Progen Biotechnik, Heidelberg, Germany). Collagen III and α-SMA were visualized with 3-amino-9-ethylcarbazole (AEC, Sigma Aldrich) as chromogen and their presence directly adjacent to the airway epithelium were quantified in the total lung section by morphometric analysis.
using Aperio ImageScope viewing software 11.2.0.780. Positively stained tissue was expressed as mm$^2$ per mm airway in the total lung section. Staining in the parenchyma or adjacent to blood vessels were excluded from analysis. Each analysis was performed blinded by the same observer, and cartilaginous airways, if present, were excluded from all measurements.

Statistical methods

The nonparametric Kruskal-Wallis test was used to assess whether differences between groups existed, followed by a Mann–Whitney $U$-test for post-hoc analysis. These analyses were performed using GraphPad Prism 5.0 and differences were considered statistically significant at two-sided $p$-values less than 0.05. $p<0.10$ was considered a statistical trend. To determine predictors of airway hyperresponsiveness (AHR), the parameters that were independent predictors of area under the curve (AUC) of airway resistance (R) in the univariate analysis for each model (HDM or FDE) were selected ($p<0.05$). When residuals were not normally distributed, appropriate log$_{10}$ transformation of the data was performed. Subsequently, a stepwise multiple linear regression model was performed to explain the variability in AUC of R, using SPSS Statistics 22 (IBM, Amsterdam, The Netherlands).

RESULTS

FDE exposure induces corticosteroid resistant lung neutrophilia

To assess the effects of FDE exposure on distribution of inflammatory cells, BALF and lung cell isolates were analyzed. In BALF, FDE-exposed mice had higher numbers of neutrophils as compared to PBS-exposed mice ($p<0.01$, figure 2A). Numbers of neutrophils remained high in FDE-exposed fluticasone propionate-treated mice ($p<0.01$ as compared to PBS). Lung cells were isolated and analyzed by flow cytometry. The number of total lung cells was higher in FDE- and HDM-exposed mice as compared to controls (figure 2B, $p<0.01$). Fluticasone propionate treatment prevented FDE and HDM-induced higher numbers of total lung cells ($p<0.05$ and $p<0.01$, respectively). Numbers of neutrophils were higher in FDE-exposed mice as compared to controls ($p<0.01$), and to a lesser extent, also higher in HDM-exposed mice ($p<0.05$, figure 2C). Fluticasone treatment failed to suppress lung neutrophilia in mice exposed to FDE or HDM.
Figure 2: FDE exposure induces corticosteroid resistant lung neutrophilia. A) Bar graph showing mean ± SEM of differential BALF cell counts. Scatter dot plots showing median and distribution of B) total isolated lung cell counts, C) numbers of neutrophils, D) macrophages and E) eosinophils in the lungs from different groups. *p<0.05, **p<0.01 from subgroup analysis (PBS versus HDM or PBS versus FDE), #p<0.05, ##p<0.01 from subgroup analysis (HDM versus HDM+FP or FDE versus FDE+FP).
Fluticasone propionate treatment dampens other inflammatory cells in the lungs

Macrophages, eosinophils and lymphocytes were not increased in BALF of FDE-exposed mice (figure 2A). HDM-exposed mice had higher numbers of eosinophils in BALF as compared to PBS-exposed mice (p<0.01). Fluticasone propionate treatment suppressed the HDM-induced lung eosinophilia (p<0.01).

Numbers of macrophages were higher in lung cell isolates of FDE- and HDM-exposed mice (p<0.01 and p<0.05, respectively), and these numbers were lower after fluticasone propionate treatment (p<0.05 and p=0.06, respectively, figure 2D). HDM-exposure induced predominantly lung eosinophilia (p<0.01) which was suppressed by fluticasone propionate treatment (p<0.01). Fluticasone treatment also prevented the higher numbers of eosinophils in FDE-treated mice, even though numbers of eosinophils were not higher in these mice as compared to controls (p=0.06, figure 2E).

Next we investigated the effects of FDE exposure and fluticasone treatment on T cells in the lungs by means of flow cytometry. FDE exposure induced higher numbers of cytotoxic T cells (p<0.01, figure 3A), activated cytotoxic T cells (p<0.01, figure 3B) and Foxp3+ cytotoxic T cells (p=0.06, figure 3C) as compared to control mice. HDM exposure induced higher numbers of cytotoxic T cells (p<0.05, figure 3A), activated cytotoxic T cells (p<0.01, figure 3B), Foxp3+ cytotoxic T cells (p=0.06, figure 3C), T helper cells (p<0.05, figure 3D), activated T helper cells (p<0.05, figure 3E) and regulatory T cells (p<0.01, figure 3F). Fluticasone propionate treatment prevented the FDE- as well as HDM-induced higher numbers of all T cell types measured, except for Foxp3+ cytotoxic T cells induced by FDE exposure.

Figure 3: Fluticasone treatment dampens T cells in the lungs. Scatter dot plots presenting median and distribution of numbers of A) T cytotoxic cells, B) activated T cytotoxic cells, C) FOXp3+ T cytotoxic cells, D) T helper cells, E) activated T helper cells and F) regulatory T cells of mice from different exposure groups
Effects of fluticasone treatment on BALF cytokines

FDE exposure led to higher levels of the Th17 cytokines IL-1β (p<0.01), IL-6 (p<0.01), IL-17A (p<0.01), CXCL1 (p<0.05); Th1 cytokines IL-12p70 (p<0.05) and TNFα (p<0.01), and also Th2 cytokines IL-4 (p<0.01), IL-5 (p<0.01) and IL-13 (p<0.05, table 2). Fluticasone propionate treatment prevented FDE-induced higher levels of IL-1β (p<0.05), IL-17A (p<0.01), CXCL1 (p<0.01) and IL-13 (p<0.05). HDM exposure induced higher levels of IL-4 (p<0.01), IL-5 (p<0.01) and IL-13 (p<0.05, table 2). Fluticasone propionate treatment prevented FDE-induced higher levels of IL-1β (p<0.05), IL-17A (p<0.01), CXCL1 (p<0.05); Th1 cytokines IL-12p70 (p<0.05) and TNFα (p<0.01), and also Th2 cytokines IL-4 (p<0.01), IL-5 (p<0.01) and IL-13 (p<0.05, table 2). Fluticasone propionate treatment prevented the higher levels of all cytokines measured, except CXCL1.

Fluticasone propionate treatment has no effect on FDE- or HDM-induced goblet cells.

Since remodeling of the airways is known to contribute to AHR, numbers of goblet cells were assessed as well as Collagen III deposition around the airways and airway smooth muscle thickness. Exposures to FDE and HDM induced higher numbers of goblet cells in the airways (p<0.01 for both exposures as compared to control mice), whereas fluticasone propionate treatment had no significant effect on the higher numbers of goblet cells (figure 4). Collagen III deposition around the airways or airway smooth muscle thickness were not different in all experimental groups (data not shown).

Figure 4: Fluticasone propionate treatment has no effect on FDE- or HDM-induced goblet cells. Scatter dot plots presenting median and distribution of numbers of goblet cells in the airways of mice from different exposure groups.
Effects of fluticasone treatment on BALF cytokines

Figure 4: Fluticasone propionate treatment has no effect on FDE- or HDM-induced goblet cells.

Since remodeling of the airways is known to contribute to AHR, numbers of goblet cells were assessed as well as Collagen III deposition around the airways and airway smooth muscle thickness. Exposures to FDE and HDM induced higher numbers of goblet cells in the airways (p<0.01 for both exposures as compared to control mice), whereas fluticasone propionate treatment had no significant effect on the higher numbers of goblet cells.

Fluticasone propionate treatment prevented the higher levels of all cytokines measured, except CXCL1.

Table 2 Th1- Th2- and Th17-related cytokines in BALF

<table>
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<tr>
<th>Cytokine (pg/ml)</th>
<th>PBS</th>
<th>HDM</th>
<th>HDM + FP</th>
<th>FDE</th>
<th>FDE + FP</th>
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<td></td>
<td></td>
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<tr>
<td>IL-12p70</td>
<td>0.06 (0.01-0.07)</td>
<td>0.15 (0.10-0.21)**</td>
<td>0.04 (0.04-0.07)##</td>
<td>0.098 (0.04-1.17)*</td>
<td>0.06 (0.04-0.01)</td>
</tr>
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<td>IFNγ</td>
<td>0.11 (0.07-0.27)</td>
<td>0.19 (0.11-0.21)</td>
<td>0.09 (0.07-0.11)#</td>
<td>0.14 (0.09-1.02)</td>
<td>0.09 (0.05-0.15)</td>
</tr>
<tr>
<td>TNFα</td>
<td>1.56 (0.68-26.68)</td>
<td>2.31 (1.31-3.10)</td>
<td>0.63 (0.54-2.31)##</td>
<td>4.98 (3.53-22.31)**</td>
<td>2.84 (0.47-5.52)</td>
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<td><strong>Th2</strong></td>
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<tr>
<td>IL-4</td>
<td>0.04 (0.01-0.06)</td>
<td>18.31 (14.48-29.95)**</td>
<td>0.85 (0.28-2.33)##</td>
<td>0.25 (0.11-1.37)**</td>
<td>0.21 (0.13-0.70)</td>
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<td>IL-5</td>
<td>0.17 (0.07-0.20)</td>
<td>10.17 (3.88-20.78)**</td>
<td>1.87 (0.80-4.82)##</td>
<td>0.93 (0.27-5.36)**</td>
<td>0.97 (0.13-3.61)</td>
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<tr>
<td>IL-13</td>
<td>0.09 (0.06-0.16)</td>
<td>10.08 (5.52-14.32)**</td>
<td>0.74 (0.31-1.13)##</td>
<td>0.22 (0.16-0.56)*</td>
<td>0.12 (0.06-0.19)#</td>
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<td>IL-10</td>
<td>0.27 (0.09-0.53)</td>
<td>12.14 (4.61-25.79)**</td>
<td>0.27 (0.18-0.44)##</td>
<td>0.18 (0.0-2.21)</td>
<td>0.18 (0.0-0.44)</td>
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<td><strong>Th17</strong></td>
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<td>IL-1β</td>
<td>0.06 (0.0-0.19)</td>
<td>0.25 (0.19-0.38)**</td>
<td>0.06 (0.0-0.13)##</td>
<td>0.48 (0.32-2.10)**</td>
<td>0.19 (0.13-0.32)#</td>
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<tr>
<td>IL-6</td>
<td>2.11 (0.17-6.08)</td>
<td>4.22 (1.52-13.90)</td>
<td>1.09 (0.50-3.54)#</td>
<td>13.06 (8.93-123-68)**</td>
<td>8.71 (3.37-14.42)</td>
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<td>IL-17A</td>
<td>0.06 (0.0-0.45)</td>
<td>0.62 (0.45-1.02)*</td>
<td>0.08 (0.03-0.31)#</td>
<td>5.69 (1.64-14.28)**</td>
<td>1.27 (0.45-2.87)#</td>
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<tr>
<td>CXCL1</td>
<td>21.62 (13.66-38.34)</td>
<td>79.74 (24.66-123.76)*</td>
<td>47.19 (34.63-96.38)</td>
<td>42.96 (23.44-98.88)*</td>
<td>16.64 (9.50-24.58)#</td>
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<td>CXCL2</td>
<td>0.64 (0.27-1.00)</td>
<td>0.51 (0.30-0.91)</td>
<td>1.04 (0.63-2.50)##</td>
<td>0.32 (0.21-1.58)</td>
<td>0.24 (0.06-0.42)</td>
</tr>
</tbody>
</table>

Data are presented as median (range). HDM: house dust mite; FP: fluticasone propionate; FDE: farm dust extract; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor.*p<0.05, **p<0.01 from subgroup analysis (PBS versus HDM or PBS versus FDE), #p<0.05, ##p<0.01 from subgroup analysis (HDM versus HDM+FP or FDE versus HDM+FP).
FDE exposure induces corticosteroid sensitive AHR

Both FDE and HDM exposures induced airway hyperresponsiveness (AHR) as calculated by the area under the curve of resistance, which was more severe in HDM-exposed mice (p=0.05 and p<0.05, respectively, figure 5 A and B). Fluticasone propionate treatment prevented the AHR in both FDE- and HDM-exposed mice (p<0.05 and p<0.01, respectively).

![Figure 5: A) Methacholine dose-response resistance curve (mean ± SEM are shown). B) Scatter dot plot showing median and distribution of area under the curve of resistance.](image)

Predictors of AHR in FDE and HDM models of lung inflammation

Since FDE and HDM induced different inflammatory profiles with AHR as a common feature, we aimed to further investigate which parameters could underlie the AHR in each model. First, we identified parameters that were independently associated with AHR in each model (p<0.05). Since remodeling markers were studied in a different set of mice than AHR, associations between remodeling parameters and AHR were not assessed. A linear regression model revealed that in the FDE model, IL-1β and IL-17 were the best predictors of AHR, explaining 77% of the variability in AHR (adjusted r square 0.77). A model with IL-1β alone accounted for 65% of the AHR. In the HDM model, TNFα, IL-6 and numbers of T helper cells accounted for 98% of the variability in AHR, while TNFα alone explained 79% of the AHR.

DISCUSSION

Asthma control is complicated by the variety in phenotypes of the disease. Currently, inhaled corticosteroid treatment is the most effective therapy for controlling asthma. Nevertheless, while corticosteroid treatment improves the quality of life of many asthmatics, patients with nonallergic or severe asthma respond poorly to this treatment (18). Several mechanisms for reduced corticosteroid responsiveness have been identified, including but not limited to, presence of increased neutrophilic inflammation (19,20).
the present study, we investigated the effects of fluticasone propionate on nonallergic asthma in mice, using an established model of FDE intranasal instillation, known to induce marked neutrophilic inflammation in the lungs (9,11,21,22). Since corticosteroid treatment is known to be effective in eosinophilic, allergic asthma, the HDM model of allergic asthma was used as a positive control for the corticosteroid treatment effect. We found that FDE induces corticosteroid resistant lung neutrophilia, as assessed in BALF and lung tissue. Intriguingly, FDE-induced high levels of Th17 cytokines CXCL1, IL-1β and IL-17A, and AHR were reduced to control level after fluticasone propionate treatment. In the allergic asthma model, corticosteroid treatment prevented all HDM-induced inflammatory markers as well as AHR.

Epidemiological studies have shown that agricultural workers have a higher risk for developing nonallergic asthma, chronic bronchitis and chronic obstructive pulmonary disease (COPD) (6,7,23). Chronic exposure to high levels of organic dusts may thus be causally involved in disease development and severity (7). Exposure of healthy human volunteers to swine barns induces AHR, pronounced airway neutrophilia, increased numbers of alveolar macrophages and increased levels of the proinflammatory cytokines TNFα, IL-6, IL-8 and IL-17 (11,12,24-26). In mice, intranasal exposure to farm dust extracts induces AHR and Th1/Th17 lung inflammation, associated with increased numbers of neutrophils and macrophages (9,16,21). The present study is the first to demonstrate that fluticasone propionate treatment effectively prevented FDE-mediated AHR and levels of IL-1β, CXCL1 and IL-17 in the lungs, while neutrophilic infiltration was resistant to fluticasone propionate treatment. In a human study, Ek and colleagues have shown that 2-week treatment of healthy volunteers with inhaled and intranasal fluticasone propionate prior to swine barn exposure, attenuated levels of TNFα and IL-8 in nasal lavage, while the treatment had no effect on AHR to methacholine induced by the exposure (27,28). The divergent results regarding AHR are probably due to differences in experimental set up. In the mentioned study, fluticasone propionate treatment was used as prevention, since healthy subjects were treated 2 weeks before exposure to swine barns, while in our study mice were treated 3 weeks after the first FDE exposure and took place for three weeks, concomitant with FDE exposures, which mimics more appropriately the situation experienced by agricultural workers.

Our finding that FDE-induced neutrophilic inflammation is resistant to fluticasone propionate treatment is in agreement with several studies in mouse models of lung disease that have shown that neutrophils respond poorly to corticosteroid treatment (14,29,30). A possible explanation for this may be the reduced glucocorticoid receptor expression in airway neutrophils (31). The proinflammatory cytokine IL-17A has also been shown to promote corticosteroid resistance (14,15,32). IL-17 is thought to contribute to asthma pathogenesis by inducing smooth muscle contraction (33), increasing mucus production (34) and stimulating the production of inflammatory cytokines, including CXC chemokines capable of recruiting neutrophils (14,32,35,36). Furthermore, IL-17 levels are increased in BALF and sputum of asthmatics and correlate with asthma severity. Indeed, we found higher levels of IL-17 in BALF of mice exposed to HDM and even higher levels in FDE-exposed mice. Interestingly, we found that both HDM- and FDE-induced higher levels of IL-17 were sensitive to fluticasone propionate treatment. This was also found for CXCL1. This data is conflicting with other studies that show that IL-17 and CXCL1 levels may be corticosteroid resistant. These divergent findings can be explained by differences in
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models used, as well as by the duration of fluticasone treatment which was longer in our study. We treated mice 4 times a week, for 3 consecutive weeks, while in other studies mice were treated twice (14) or epithelial cells for 2-24 hours (15,32). It is possible that IL-17 and CXCL1 are less sensitive to corticosteroid treatment as compared to Th2 cytokines, but that in the long term, these differences are not noticed anymore. Noteworthy, the lack of correlation between neutrophil counts in BALF and lung tissue and levels of its attracting chemokine CXCL1 in BALF highlight the complexity of the inflammatory response in this model and the elaborated interplay between the various cytokines and chemokines that control cellular influx into the lung. Along these lines, IL-17 and CXCL1 may be important in induction of neutrophil influx to the lungs but may not be required for sustained neutrophil inflammation. Levels of other chemokines involved in neutrophil recruitment, such as CCL2 and CXCL10 or the growth factor granulocyte-colony stimulating factor (G-CSF) (37) were not assessed in this study.

In the HDM model, the most prominent inflammatory cell type was, by far, the eosinophil, but neutrophil counts were also higher, in agreement with other studies (38). Interestingly, numbers of neutrophils were higher in HDM-exposed mice that were treated with fluticasone propionate as compared to HDM-exposed mice without treatment. This confirms previous findings that corticosteroid treatment inhibits neutrophil apoptosis, promoting survival of functional neutrophils (20,39), and may contribute to leukocyte infiltration in the lungs in certain models (40). We speculate that this was not observed in the FDE group due to a plateau effect, since the numbers of neutrophils are much higher in this group than in the HDM group.

Besides lung neutrophilia, the FDE model induced a remarkable increase in numbers of macrophages in lung tissue. We have recently shown that although numbers of macrophages are higher in both HDM and FDE models, the functional phenotype of macrophages is distinct in each model (16). Numbers of macrophages were however not higher in BALF of FDE-exposed mice, as compared to controls. This highlights the relevance of considering different compartments of the lung (e.g. airways and parenchyma), especially regarding the study of alveolar and interstitial macrophages in future studies. Furthermore, FDE-exposed mice showed increased numbers of cytotoxic T cells, which is in agreement with our previous findings in peripheral blood mononuclear cells of agricultural workers in which we showed that these cytotoxic cells expressed IL-17 and IFNγ (9).

With exception of neutrophils, fluticasone propionate treatment strongly suppressed numbers of other inflammatory cells in both models studied. This was noticeable in total lung cells, eosinophils, macrophages and T cells. Corticosteroids exhibit their potent anti-inflammatory effect by binding to the glucocorticoid receptor, which then translocates to the nucleus where it represses many pro-inflammatory genes and activate anti-inflammatory genes. As a consequence, corticosteroids suppress the production of certain chemotactic mediators, inhibiting the recruitment of inflammatory cells to the airways, including eosinophils, T cells, mast cells and dendritic cells (18), while other chemokines may be induced, resulting in leukocyte recruitment to the lungs (40). Recent studies have shown that corticosteroids do not generally suppress the immune system, but rather cause a shift in the innate-adaptive balance of the immune response (40).

Regarding asthma symptoms and AHR, a reduced response to corticosteroid treatment has been associated with more neutrophilic inflammation (19,20). Green and
collaborators showed in adult asthmatics that a subgroup with more neutrophils in sputum had an impaired response to a two-month corticosteroid treatment regarding symptoms, forced expiratory volume in 1 second (FEV₁) and AHR to methacholine, as compared to asthmatics with more eosinophils in sputum (19). Although patients with eosinophilic asthma respond better to corticosteroid treatment, it has been shown that a proportion of patients with neutrophilic or paucigranulocytic asthma showed significant improvement in symptoms and AHR after fluticasone propionate treatment (20). Smoking asthmatics, which also have more neutrophils and less eosinophils in sputum and blood, have a blunted short term response to fluticasone propionate treatment. However, after one year of treatment, these patients presented a similar response as never smokers to fluticasone propionate treatment regarding lung function, even though numbers of neutrophils in sputum remained high in smokers (41), showing a disparity between neutrophilic inflammation and lung function. COPD patients also show improvement in AHR after smoking cessation, albeit high number of neutrophils in sputum (42). Such findings are compatible with our data showing that AHR was suppressed by fluticasone treatment despite sustained high numbers of neutrophils in BALF and lung tissue.

To further assess which parameters could predict AHR in each model of lung inflammation, a linear regression model was used. In the FDE model, IL-1β and IL-17 were the best predictors of AHR, while in the HDM model, the proinflammatory cytokines involved in systemic inflammation, TNFα and IL-6, were the best predictors of AHR. IL-1β is known to be involved in the differentiation of Th17 cells and has also been implicated in AHR development in animal models (43,44). A positive correlation between IL-17 levels in sputum and AHR to methacholine in asthma patients has been reported (45). Furthermore, it has recently been shown that IL-17A produced by Th17 cells drives HDM- and ovalbumin-induced AHR in mice and exerts a direct effect on airway smooth muscle cells from mice and men, increasing contractibility (33). An association between levels of TNFα in induced sputum and AHR in asthmatic subjects has been previously shown (46). TNFα promotes an increase in the contractility of human bronchial tissue in vitro, thereby possibly inducing AHR (47). Regarding IL-6, serum levels of this cytokine were shown to be higher in asthmatics in comparison to healthy controls and even higher during exacerbations (48). Our data support findings in the literature and demonstrate that in mouse models of different types of asthma, different inflammatory mediators predict AHR the best.

In conclusion, our results show that fluticasone propionate treatment suppressed FDE-induced AHR, inflammatory cytokines and inflammatory cells, except for the number of neutrophils, which was corticosteroid resistant. In nonallergic asthma IL-1β and IL-17 contribute to severity of AHR, and could thus be attractive targets for improved treatment of this asthma phenotype.

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